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### Permalink

<https://escholarship.org/uc/item/5wx2j9v0>

### Journal

The Journal of experimental medicine, 173(6)

### ISSN

0022-1007

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### Publication Date

1991-06-01

### DOI

10.1084/jem.173.6.1483

Peer reviewed

# The Binding Affinity of Human IgG for its High Affinity Fc Receptor Is Determined by Multiple Amino Acids in the C<sub>H</sub>2 Domain and Is Modulated by the Hinge Region

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## Summary

A family of chimeric immunoglobulins (Igs) bearing the murine variable region directed against the hapten dansyl linked to human IgG1, -2, -3, and -4 has been characterized with respect to binding to the human high affinity Fc  $\gamma$  receptor, Fc $\gamma$ R<sub>1</sub>. Chimeric IgG1 and -3 have the highest affinity association ( $K_a = 10^9 \text{ M}^{-1}$ ), IgG4 is 10-fold reduced from this level, and IgG2 displays no detectable binding. A series of genetic manipulations was undertaken in which domains from the strongly binding subclass IgG3 were exchanged with domains from the nonbinding subclass IgG2. The subclass of the C<sub>H</sub>2 domain was found to be critical for determining IgG receptor affinity. In addition, the hinge region was found to modulate the affinity of the IgG for Fc $\gamma$ R<sub>1</sub>, possibly by determining accessibility of Fc $\gamma$ R<sub>1</sub> to the binding site on Fc. A series of amino acid substitutions were engineered into the C<sub>H</sub>2 domain of IgG3 and IgG4 at sites considered potentially important to Fc receptor binding based on homology comparisons of binding and nonbinding IgG subclasses. Characterization of these mutants has revealed the importance for Fc $\gamma$ R<sub>1</sub> association of two regions of the genetic C<sub>H</sub>2 domain separated in primary structure by nearly 100 residues. The first of these is the hinge-link or lower hinge region, in which two residues, Leu(234) and Leu(235) in IgG1 and -3, are critical to high affinity binding. Substitution at either of these sites reduces the IgG association constant by 10–100-fold. The second region that appears to contribute to receptor binding is in a hinge-proximal bend between two  $\beta$  strands within the C<sub>H</sub>2 domain, specifically, Pro(331) in IgG1 and -3. As a result of  $\beta$  sheet formation within this domain, this residue lies within 11 Å of the hinge-link region. Substitution at this site reduces the Fc receptor association constant by 10-fold.

The antibody molecule serves as an immunologic bridge, combining in one polypeptide sites responsible for recognition of foreign pathogens and others responsible for triggering of host response systems. IgG, the predominant form of serum antibody, interacts through its Fc region with a variety of host effector molecules, among which are a group of surface receptors found on cells predominantly of hemopoietic origin. This Fc receptor family can be divided based on IgG subclass specificity and mAb recognition into three groups: Fc $\gamma$ R types I, II, and III. Fc $\gamma$ R<sub>1</sub> is distinctive in being the only receptor capable of binding monomeric IgG with high affinity (1).

The human Fc $\gamma$ R<sub>1</sub> receptor binds ligand with the subclass preference: IgG1, -3 > IgG4 >> IgG2 (2). It binds murine IgG2a, but not muIgG1 or muIgG2b, with affinity equivalent to that of human IgG (3). It has a valence for IgG of one (4), and association of IgG with huFc $\gamma$ R<sub>1</sub> appears to be

mediated by a single heavy chain (5), although there are two binding sites on each IgG molecule. Fc $\gamma$ R<sub>1</sub> functions demonstrated in vitro include phagocytosis (6), and antibody-dependent cellular cytotoxicity (ADCC)<sup>1</sup> (7–11). Evidence supports a unique role for Fc $\gamma$ R<sub>1</sub> early in the immune response before antigen-specific IgG reaches high levels.

Early attempts to localize the site on IgG responsible for associating with huFc $\gamma$ R<sub>1</sub> using proteolytic fragments of Ig were inconclusive (12, 13). Analysis of mutant Igs was more informative and suggested that at least a number of the residues required for association are located in the C<sub>H</sub>2 domain, potentially in the NH<sub>2</sub>-terminal end of the domain (3).

Examination of a number of IgG subclasses for amino acids

<sup>1</sup> Abbreviations used in this paper: Ab, antibody; ADCC, antibody-dependent cellular cytotoxicity; EE, exon exchange; SDM, site-directed mutagenesis.

conserved among strong ( $K_a > 10^8 \text{ M}^{-1}$ ), but not among weak binders ( $K_a < 10^6 \text{ M}^{-1}$ ) revealed two homology regions potentially involved in  $\text{Fc}\gamma\text{R}_1$  binding. The first is a hexapeptide sequence in the hinge-link region immediately before the  $\beta$  strand formation of the  $\text{C}_{H2}$  domain. This peptide segment, conserved among huIgG1 and -3, muIgG2a, and rabbit IgG, consists of the sequence Leu - Leu - Gly - Gly - Pro - Ser (residues 234-239, EU numbering [14]). In huIgG4, an intermediate affinity IgG, the sequence contains a single amino acid substitution: Phe for Leu at position 234. Murine IgG2b, a weakly binding IgG, likewise contains a single residue substitution: Glu for Leu at position 235. In human IgG2, which shows no binding activity, there are two substitutions and a deletion leading to the sequence Val(234) - - - - Ala(236) - Gly - Pro - Ser (Fig. 1). The significance of this region in mediating binding to the high affinity  $\text{Fc}$  receptor was recently demonstrated by Duncan et al. (15), who reported conferring IgG2a-like binding properties on an IgG2b transfectoma protein by using oligonucleotide-directed mutagenesis to change Glu(235) to Leu.

A second region of the  $\text{C}_{H2}$  domain identified by homology as a potential contributor to  $\text{Fc}\gamma\text{R}_1$  binding is located near the COOH-terminal end of the domain's primary structure. This broad stretch of residues extends over two of the three  $\beta$  strands in the Y-face of the domain, including a hinge-proximal bend between the strands, and contains 16 residues perfectly conserved among strongly binding IgGs. Of particular interest is the fact that among the four human IgG subclasses, there is only one substituted site within these conserved residues: a pair of serine residues in IgG4 replaces Ala(330) and Pro(331) (Fig. 1) (present in the other three subclasses); this difference is located in the bend between the two  $\beta$  strands and is predicted to fold into close proximity to the hinge-like region, although the latter is not strictly resolved in the crystallographic  $\text{Fc}$  model due to a high degree of disorder in this region of the crystal (16).

We have taken two approaches to defining the structural features of IgG that influence, either directly or via conformational effects, the molecule's association with the human  $\text{Fc}\gamma\text{R}_1$ . To examine the contributions made by individual domains and by the hinge, gene manipulation has been used to exchange exons between the four cloned human IgG constant region genes. Expression of these genes in a chimeric antidansyl system in association with the human  $\text{C}_\kappa$  light chain allowed the examination of intact human Ig "variant" proteins with a series of domain interchanges that do not suffer from the drawbacks of the proteolytic cleavage studies or the uncertainties of heterologous IgG receptor systems. Studies of natural variant proteins to date had indicated a role for  $\text{C}_{H2}$  in the binding to  $\text{Fc}\gamma\text{R}_1$ , but have not ruled out a contribution from the  $\text{C}_{H3}$  domain, nor had the role of the hinge been addressed, beyond establishing that its presence is required for full strength binding.

On a sub-domain level, oligonucleotide-directed site-specific mutagenesis has been used within the  $\text{C}_{H2}$  domain to better define the amino acid residues that account for the  $\text{Fc}\gamma\text{R}_1$  affinity differences between IgG subclasses. By this approach,

we have examined residues at the  $\text{NH}_2$ - and COOH-terminal ends of the domain sequence indicated by homology patterns to be potentially important for receptor binding. These mutant IgG genes, like those of the domain-altered proteins, have been expressed in the context of the chimeric antidansyl system in order that homologous (human) receptor IgG interactions may be investigated. These studies have indicated the importance of residues at both ends of the  $\text{C}_{H2}$  domain linear sequence in either directly contacting the receptor, or maintaining the functional conformation of the binding site (or both), and have shown that the hinge modulates the affinity of IgG for  $\text{Fc}\gamma\text{R}_1$ .

## Materials and Methods

**Vectors.** The Sall-BamHI cassette containing the constant region gene was subcloned into either pBR322 for exon exchange (EE), or M13mp19 for site-directed mutagenesis (SDM). EE was performed using appropriate intronic restriction sites (e.g., PvuI), and was confirmed by restriction digest analysis and double-stranded DNA sequence analysis. SDM was performed by the double-primer method (17) using single-mismatch oligonucleotides, and was confirmed by sequence analysis. Mutated constant region genes were cloned as Sall-BamHI fragments into the mammalian expression vector pSV2 $\Delta\text{Hgpt}$  (18) with the expressed  $\text{V}_H$  gene from a mouse anti-DNS hybridoma (Fig. 2).

**Transfectoma Production.** The heavy chain was transfected into the nonproducing myeloma cell line P3X63AG8.653 along with the chimeric anti-DNS  $\kappa$  light chain gene carried in the compatible expression vector pSV184 $\Delta\text{Hneo}$  by protoplast fusion, as previously described (18). Transfectants were selected with G418 at 1.0 mg/ml, and surviving clones screened for antibody (Ab) production by ELISA using DNS/BSA-coated plates. The amount of bound chimeric Ab was determined using alkaline phosphatase-conjugated polyclonal goat Ab (Sigma Chemical Co., St. Louis, MO) against human IgG constant regions. Clones producing large quantities of anti-DNS Ab were expanded and maintained in IMDM containing 5% calf serum.

**Characterization of Chimeric Ab.** To characterize the assembly, secretion, and molecular weight of Ig, cells were labeled with  $^{35}\text{S}$ -methionine ( $^{35}\text{S}$ -met). Ab molecules in the supernatants were immunoprecipitated with polyclonal rabbit Ab against human Fc and *Staphylococcus aureus* protein A (IgG sorb; The Enzyme Center, Malden, MA) and analyzed by SDS-PAGE. Antibodies were purified from culture supernatants as previously described (19).

**FcR Binding.** An enzymatic assay was developed to quantitate the binding affinities of chimeric Ab for  $\text{Fc}\gamma\text{R}_1$  (20). Human monocyte-like U937 cells (2) were incubated with chimeric IgG molecules at various concentrations. After a 2-h incubation,  $\beta$ -galactosidase-conjugated DNS was added and incubated for another 2 h. The cells were then washed by centrifugation through a sucrose pad and  $\beta$ -galactosidase bound to IgG quantitated using the substrate *o*-nitrophenyl galactoside. Scatchard analysis (21) was used to determine the association constant of IgG to the receptor and the number of receptors per cell.

## Results

**Antibody Characterization.** The size of the H and L chains produced by the transfectants and their assembly into  $\text{H}_2\text{L}_2$  tetramers was assessed by metabolic protein labeling, followed

Hinge Link Region							Hinge-Proximal Bend						
234	235	236	237	238	239		328	329	330	331	332	333	
Leu	Leu	Gly	Gly	Pro	Ser	...	Leu	Pro	Ala	Pro	Ile	Glu	IgG1,3
<b>Phe</b>	Leu	Gly	Gly	Pro	Ser	...	Leu	Pro	<b>Ser</b>	<b>Ser</b>	Ile	Glu	IgG4
Leu	<b>Glu</b>	Gly	Gly	Pro	Ser	...	Leu	Pro	<b>Ser</b>	Pro	Ile	Glu	IgG2b
<b>Val</b>	---	<b>Ala</b>	Gly	Pro	Ser	...	Leu	Pro	Ala	Pro	Ile	Glu	IgG2

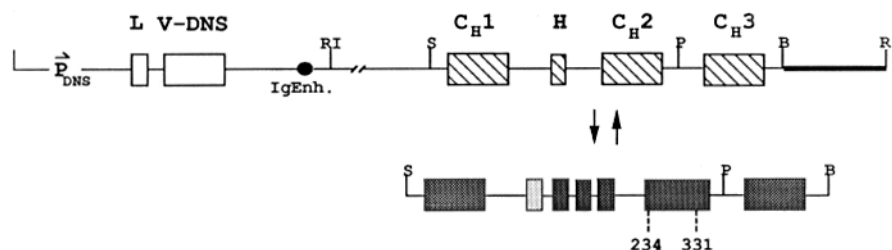
**Figure 1.** Amino acid sequence alignment from the C<sub>H</sub>2 domains of human IgG1, -2, -3, -4, and murine IgG2a and -2b. Two regions within C<sub>H</sub>2 are compared across subclasses: the hinge-link or lower hinge region (residues 234–239) and a hinge-proximal bend between two  $\beta$  strands in the Y-face (residues 328–333). Each amino acid is indicated below its corresponding residue number (EU system); residues differing from high affinity (hulG1, -3, and mulG2a) sequence are highlighted in bold italics.

by immune precipitation from culture supernatants, and separation of polypeptides by SDS-PAGE. The wild-type IgG 1–4 have been analyzed previously (22) and shown to correspond in mobility to their expected molecular weights. The H chain of IgG3 is larger than those of the other subclasses due to its extended hinge length. IgG1, -2, and -3 are secreted as covalently joined H<sub>2</sub>L<sub>2</sub> molecules with little evidence of assembly intermediates in the secretions. IgG4, however, is secreted as both H<sub>2</sub>L<sub>2</sub> and as HL “half molecules”, a feature of this subclass apparently determined by the hinge region, since it is transferable by genetic exchange of the hinge exon (22).

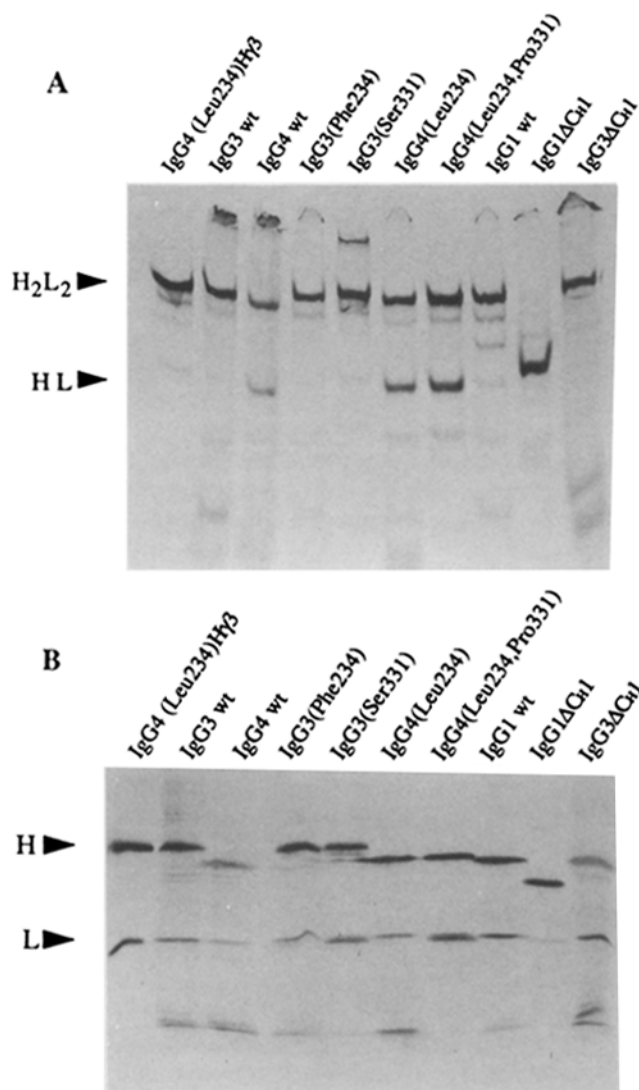
The hybrid IgGs made by exon exchange/deletion and the IgG point mutations all displayed mobilities on SDS-PAGE consistent with their molecular weights, indicating that no gross defects of transcript splicing or frameshift resulted from the genetic manipulations. A representative autoradiogram of metabolically labeled mutant IgGs is shown in Fig. 3 (A, nonreducing conditions; B, reducing conditions). As expected, all molecules bearing hinge regions of the  $\gamma_4$  subclass (IgG4, IgG4[Leu235], IgG4[Leu235, Pro331]) are secreted with a significant fraction as HL dimers. Because these form H<sub>2</sub>L<sub>2</sub> tetramers by noncovalent interactions, attempts to purify away half molecules by gel filtration were unsuccessful. No attempt was made to separate these species under denaturing conditions due to concern over the effects of such treatment on IgG effector function activity.

Of particular interest with respect to assembly were the two IgG molecules deleted for C<sub>H</sub>1, since they might be expected to be severely compromised conformationally. As is evident under reducing conditions (Fig. 3 B), both IgG1 $\Delta$ C<sub>H</sub>1 and IgG3 $\Delta$ C<sub>H</sub>1 make H chains with the expected decrease in size from their wild-type counterparts. From Fig. 3 A, it appears that IgG1 $\Delta$ C<sub>H</sub>1 is secreted primarily in the form of H<sub>2</sub> and L, whereas IgG3 $\Delta$ C<sub>H</sub>1 appears to assemble normally, secreting predominantly H<sub>2</sub>L<sub>2</sub> tetramers. All transfectants not represented in Fig. 3 make H and L chains of the anticipated size and secrete only full H<sub>2</sub>L<sub>2</sub> covalent tetramers.

**Binding of Wild-type IgG Subclasses to Fc $\gamma$ R<sub>1</sub>.** In initial experiments, the equilibrium binding constants of the four wild-type chimeric IgG subclasses to Fc $\gamma$ R<sub>1</sub> on the human monocytic cell line U937 were determined (Fig. 4 and Table 1). Fig. 4 shows representative Scatchard plots for IgG1, -3, and -4 (each curve represents a single assay). In Fig. 4 A, the binding of IgG3 to U937 cells pretreated with human IFN- $\gamma$  (100 U/ml, 18 h), which increases the expression of the Fc $\gamma$ R<sub>1</sub>, is compared with the binding of the same protein to untreated cells. Treatment with IFN- $\gamma$  causes a shift in the Scatchard curve toward the right, increasing the  $\alpha$ -intercept of the plot, in this case from 3,400 to 19,000 (representing an increase in receptor number per cell); the slope of the Scatchard plot, on the other hand, remains unchanged by treatment with IFN- $\gamma$ , indicating that there is no effect on the affinity of the IgG receptor interaction. In Fig. 4 B, the Scatchard plots of IgG1 and IgG3 assayed at different times on IFN- $\gamma$ -treated cells are compared in order to demonstrate the equivalent binding affinities of the two proteins. The apparent number of receptors per cell was seen to vary between assays even when the same protein preparation was assayed repeatedly, presumably as a consequence of uncontrolled variation in cell culture conditions. In Fig. 4 C, the binding of IgG3 and IgG4 to untreated U937 cells is compared in order to demonstrate the reduced affinity of IgG4 for Fc $\gamma$ R<sub>1</sub> (represented by the slope of the Scatchard curve). It is worth noting that although the plot for IgG4 appears nearly flat, this is a function of the dimensions of the  $y$ -axis (chosen in order that both curves could be represented in one plot); in fact, the slope is only 10-fold reduced



**Figure 2.** Limited restriction map of heavy chain construct used for expression of antidansyl chimeric antibodies. IgG2 constant region, hatched; IgG3 constant region, dark stippled, except the first, unique hinge exon, distinguished by light stipple; L, exon for hydrophobic signal peptide; V-DNS, exon for variable region domain; C<sub>H</sub>1–3, exons for heavy chain constant region domains; H, hinge exon; PDNS, endogenous murine Ig promoter; IgEnh, murine Ig heavy chain enhancer, 234, 331, sites of oligonucleotide-directed mutagenesis. Restriction sites: RI, EcoRI; S, SalI; P, PvuII; B, BamHI. The vector pSV2 $\Delta$ Hgtt is represented by a thickened line. The drawing is not to scale.



**Figure 3.** SDS-PAGE analysis of  $^{35}\text{S}$  methionine-labeled chimeric IgGs. Secreted Ig proteins were metabolically labeled with  $^{35}\text{S}$ -Methionine (15  $\mu\text{Ci}/\text{ml}$ ) as described, then immunoprecipitated using rabbit anti-human IgG Fc and *S. aureus* Protein A. (A) SDS-PAGE analysis of nonreduced IgGs on a 5% acrylamide gel. Each lane is labeled above, and the migration positions of whole IgG ( $\text{H}_2\text{L}_2$ ) and half-molecules (HL) are indicated to the side of the gel. (B) SDS-PAGE analysis of reduced IgGs on a 12.5% acrylamide gel. Lanes are labeled as in A, and the migration positions of heavy (H) and light (L) chains are indicated to the side of the gel.

from that of the curve for IgG3. IgG2 is not depicted in Fig. 4 because no binding of this subclass was detected at concentrations  $>100$ -fold higher than those used to assay IgG1. Given the sensitivity of this assay, we estimate the  $K_a$  of IgG2 to be  $<10^6 \text{ M}^{-1}$ , at least 1,000-fold reduced from the IgG1 level. Association constants for the wild-type IgGs are given in Table 1 and represent the mean of at least three independent assays. IgG1 and IgG3 show the highest affinity for  $\text{Fc}\gamma\text{R}_1$ , with  $K_a$  values of  $10^9 \text{ M}^{-1}$ . IgG4 is  $\sim 10$ -fold reduced in binding activity, and IgG2 shows no detectable binding by this assay ( $K_a < 10^6 \text{ M}^{-1}$ ). These values agree

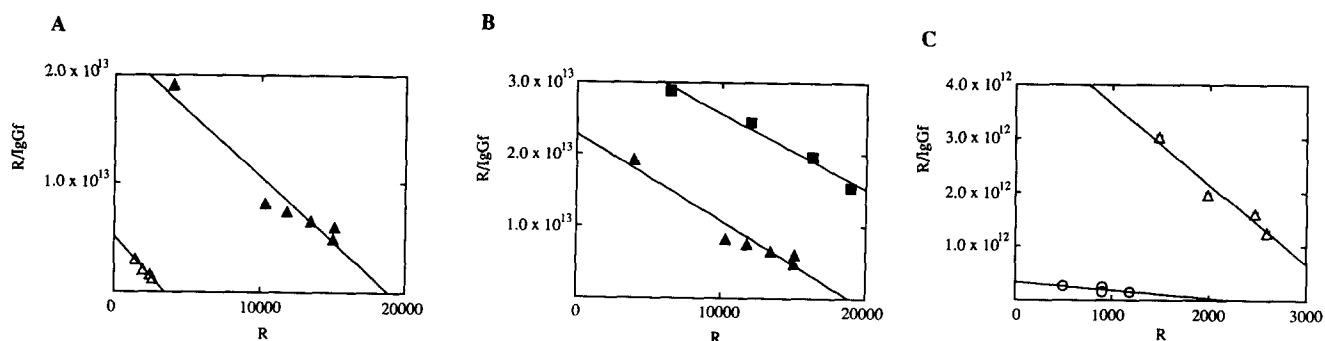
**Table 1.** Equilibrium Binding Constants of Domain-exchanged Chimeric IgGs

Immunoglobulin	Structure	Binding Constant $K_a (\text{M}^{-1})$
	$\text{VH} \text{---} \text{CH1} \text{---} \text{CH2} \text{---} \text{CH3}$	
IgG1		$1.2 (\pm 0.3) \times 10^9$
IgG2		$<1 \times 10^6$
IgG3		$1.2 (\pm 0.5) \times 10^9$
IgG4		$1.4 (\pm 0.2) \times 10^8$
2-2-2-3		$<1 \times 10^6$
3-3-3-2		$1.8 (\pm 0.2) \times 10^9$
2-2-3-2		$2.8 (\pm 1.1) \times 10^8$
3-3-2-2		$<1 \times 10^6$
3-3-2-3		$<1 \times 10^6$
IgG3 $\Delta\text{H}$		$<1 \times 10^6$
IgG1 $\Delta\text{C}\mu 1$		$3.0 (\pm 1.0) \times 10^7$
IgG3 $\Delta\text{C}\mu 1$		$2.0 (\pm 1.2) \times 10^8$
IgG3h <sub>1</sub>		$2.2 (\pm 1.3) \times 10^8$
IgG3h <sub>1(2,3,4)2</sub>		$1.8 (\pm 0.8) \times 10^8$

IgG1–4, wild-type antidansyl chimeric antibodies. Mutants with exon exchanges are given a four-digit name: the first digit refers to the subclass of the  $\text{C}\mu 1$  domain, and the second, third, and fourth digits indicate the subclasses of the hinge,  $\text{C}\mu 2$ , and  $\text{C}\mu 3$  domains, respectively. IgG3  $\Delta\text{H}$ , hinge-deleted IgG3; IgG3h<sub>1</sub>, IgG3 bearing a hinge encoded by the first (unique)  $\gamma 3$  hinge exon; IgG3h<sub>1(2,3,4)2</sub>, IgG3 with extended hinge (containing a duplication of the three reiterated  $\gamma 3$  hinge exons). The structure of each recombinant antibody is shown diagrammatically. Each  $K_a$  value represents the mean of at least three independent trials ( $\pm \text{SD}$ ).  $\text{V}_h$ , exon for the heavy chain variable domain directed against the hapten dansyl.

closely with previous reports in which the binding constants were determined using  $^{125}\text{I}$ -IgG (23, 24).

**Binding of Exon Exchange Mutant to  $\text{Fc}\gamma\text{R}_1$ .** The role of the constant region domains in  $\text{Fc}\gamma\text{R}_1$  binding was evaluated by exchanging these regions between IgG2 and IgG3 (which display opposite extremes of binding activity). Although the presence of a  $\text{C}\mu 3$  domain is required for  $\text{Fc}$  receptor binding, as indicated by the fact that a myeloma protein lacking this domain shows no affinity for  $\text{Fc}\gamma\text{R}_1$  (25), this domain does not appear critical in determining the isotype-specific binding pattern (Table 1). The proteins denoted 2223 and 3332 represent the products of  $\text{C}\mu 3$  exon exchange between IgG2 and IgG3 subclasses. IgG2 bearing the  $\text{C}\mu 3$  domain of IgG3 (2223) showed no detectable binding to U937 cells ( $K_a < 10^6 \text{ M}^{-1}$ ). The reciprocal exchange, IgG3 bearing the  $\text{C}\mu 3$  domain of IgG2 (3332), bound to U937 cells with a  $K_a$  equivalent to that of IgG3 ( $1.8 \times 10^9 \text{ M}^{-1}$ ), indicating that the  $\text{C}\mu 3$  domain is not responsible for the subclass-specific affinity difference between nonbinding IgG2 and the strongly binding IgG3. Although the presence of a  $\text{C}\mu 3$  domain is required for maximal binding  $\text{Fc}\gamma\text{R}_1$  (as discussed earlier), the subclass of this domain does not determine the



**Figure 4.** Scatchard plots of the binding of wild-type IgG to U937 cells. (A) The effect of human IFN- $\gamma$  on binding of IgG3. Binding was assayed on untreated cells (open triangles) and on cells treated with 100 U/ml of human rIFN- $\gamma$  for 18 h (filled triangles). IFN- $\gamma$  increases the number of Fc receptors per cell (represented by  $x$ -intercept) but does not affect the affinity of interaction (represented by the absolute value of the slope in each plot). In this case untreated cells yielded 3,400 receptor sites per cell; IFN- $\gamma$ -treated cells, 19,000 site per cell. (B) Comparison of IgG1 and IgG3 binding by IFN- $\gamma$  treated U937 cells. IgG1, filled squares; IgG3 filled triangles. These two proteins display equivalent affinities for Fc $\gamma$ R<sub>1</sub> (parallel Scatchard plots); the variance in receptor number estimated per cell (IgG1, 34,000; IgG3, 19,000) is not uncommon between assays and is most likely due to differences in cell culture conditions immediately before assay. (C) Comparison of IgG3 and IgG4 binding to U937 cells not treated with IFN- $\gamma$ . The decreased affinity of IgG4 for Fc $\gamma$ R<sub>1</sub> relative to IgG3 is represented by the difference in slopes of the two plots. IgG3, open triangles; IgG4, open circles. R, number of IgG molecules bound per cell; R/IgG<sub>f</sub>, the ratio of IgG molecules bound to free IgG.

isotype-specific binding phenotype, suggesting that either this domain does not directly interact with the Fc receptor or that the interacting residues are conserved between these subclasses.

By contrast, the subclass of the C<sub>H</sub>2 domain does appear to determine, by a first approximation, the binding phenotype of the molecule. Hence, IgG3 with an Fc region almost completely from IgG2 (3322), and IgG3 bearing only the C<sub>H</sub>2 domain of IgG2 (3323), show no detectable binding to U937 cells. Moreover, IgG2 with a  $\gamma_3$  C<sub>H</sub>2 domain (2232) binds Fc $\gamma$ R<sub>1</sub> with an affinity nearly that of IgG3 ( $K_a = 2.8 \times 10^8$  M<sup>-1</sup>). Interestingly, the  $K_a$  value for the protein 2232 was slightly but reproducibly lower than that of 3332 (three-fold decreased) suggesting that the hinge and/or C<sub>H</sub>1 domain may contribute to overall IgG affinity, possibly through conformational effects on the C<sub>H</sub>2 domain. Deletion of C<sub>H</sub>1 altogether from IgG1 results in a decrease in association constant of  $\sim 30$ -fold (see Table 2), whereas the equivalent deletion from IgG3 results in only a 10-fold reduction in  $K_a$ .

The hinge region plays a role both as a spacer region between the Fab and Fc regions and in imparting the molecule with segmental flexibility. The presence of the hinge region is necessary for the interaction of IgG Fc $\gamma$ R<sub>1</sub>, and hinge-deleted human IgG1 myeloma proteins Dob and Lec display little binding to the mouse analogue of this receptor (26). IgG3 contains an extended hinge of 62 amino acids, encoded genetically by four exons (one unique, followed by three reiterated exons). As the subclass with the greatest degree of segmental motion in the Fab region, IgG3 may require the extended hinge in order to prevent steric hindrance to close approach of effector molecules. As shown in Table 1, deletion of the entire hinge (IgG3 $\Delta$ H) results in complete loss of binding to the human Fc $\gamma$ R<sub>1</sub>, in agreement with the studies of Dob and Lec. Partial replacement of the hinge with a 17-residue segment encoded by the first (unique) IgG3 hinge

exon results in restoration of receptor binding, however, this protein (IgG3h<sub>1</sub>) displays a  $K_a$  value 10-fold reduced from wild-type ( $K_a = 2.2 \times 10^8$  M<sup>-1</sup>) (Table 1). Therefore, IgG3h<sub>1</sub>, which exhibits segmental flexibility equivalent to wild-type IgG3, is reduced in ability to fix complement and bind Fc $\gamma$ R<sub>1</sub> supporting a role for the extended hinge in preventing steric hindrance. Somewhat surprisingly, IgG3 bearing a hinge extended by duplication of its reiterated exons (IgG3h<sub>1(2,3,4,2)}</sub>) also exhibits  $\sim 10$ -fold reduced affinity for Fc $\gamma$ R<sub>1</sub> relative to IgG3; therefore, there is an optimal spacing between Fab and Fc.

**Amino Acid Substitutions.** Having established the importance of the C<sub>H</sub>2 domain of IgG in the interaction with Fc $\gamma$ R<sub>1</sub> on U937, we next investigated the contributions to

**Table 2.** Equilibrium Binding Constants of Amino Acid-substituted Chimeric IgGs

IgG	$K_a$
	M <sup>-1</sup>
IgG3(Glu[235])	$<1 \times 10^7$
IgG3(Phe[234])	$1.9 (\pm 0.8) \times 10^8$
IgG4(Leu[234])	$3.8 (\pm 1.4) \times 10^8$
IgG3(Ser[331])	$3.1 (\pm 2.6) \times 10^8$
IgG4(Leu[234], Pro[331])	$1.3 (\pm 0.4) \times 10^8$
IgG4(Leu[234])H $\gamma_3$	$1.0 (\pm 0.2) \times 10^8$

The name of each mutant indicates its subclass, the position of the amino acid substitution (EU number system), and the amino acid created at that site. IgG4 (Leu[234]) H $\gamma_3$  represents IgG4 substituted at position 234 (Leu for Phe) and bearing the hinge of IgG3. Each  $K_a$  value, represents the mean of at least three independent trials ( $\pm$  SD).



binding made by selected residues within the domain, using oligonucleotide-directed mutagenesis to produce amino acid substitutions. Table 2 lists a series of site-specific mutant IgG proteins and the associated  $K_a$  values for binding U937 cells.

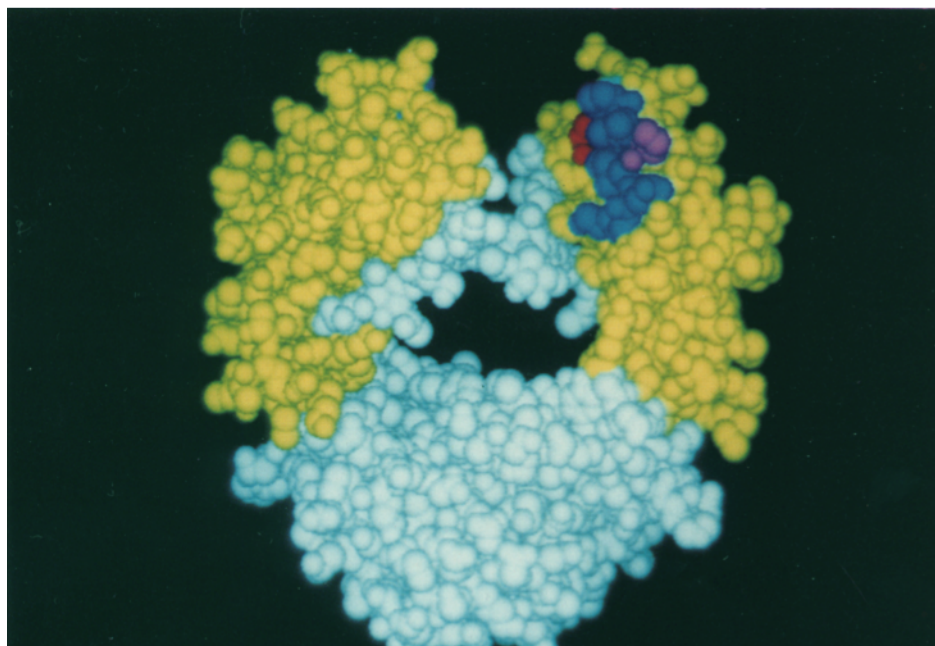
The first substitutions introduced were designed to examine the role of the homology region (identified by Woof et al. [25]) encoded at the 5' end of the  $C_H2$  exon, and falling in the hinge-link region of the molecule (Fig. 1). As discussed earlier, this conserved hexapeptide consisting of Leu(234) - Leu - Gly - Pro - Ser(239) is present in human IgG1 and IgG3, is substituted once at residue 234 by Phe in IgG4, and once at residues 235 by Glu in muIgG2b. A single amino acid substitution in the hinge-link region of mouse IgG2b, Glu(235) to Leu, increased the binding of this Ig >100-fold, to a level equivalent to that of human IgG1 (15). We have found that the reciprocal substitution of Glu for Leu(235) in IgG3 leads to a significant decrease in  $Fc\gamma R_1$  affinity (Table 2). However, our studies make it clear that in human IgG more than one residue determines Fc receptor binding affinity, and that the critical residues are not all contiguous in the primary sequence.

Human IgG4 differs in the hinge-link region from the high affinity subclasses (IgG1 and -3) at only one position: Phe replaces Leu(234). To evaluate the role played by this amino acid in determining the relatively low binding affinity of IgG4 for the receptor, the reciprocal substitution of Leu and Phe was performed between IgG3 and IgG4 at this position. IgG3 (Phe234) is reduced in association constant (Table 2) to the level of IgG4 ( $K_a = 1.9 \times 10^8 M^{-1}$ ), indicating that residue 234, like residue 235, is important in receptor binding. The reciprocally substituted protein, IgG4 (Leu[234]) exhibits increased affinity for  $Fc\gamma R_1$ , however, the binding affinity does not reach the level of IgG3 (Table 2). Apparently, other

aspects of the IgG4 molecule, potentially masked by the negative contribution of Phe(234), affect antibody receptor interactions. Sequence comparison reveals that among the  $C_H2$  residues conserved in strongly binding IgG subclasses (human IgG1, -3, mouse IgG2a), IgG also differs at residues 330 and 331. These residues are within a hinge-proximal bend between two  $\beta$  strands of the  $C_H2$  domain predicted to fold in close proximity to the hinge-link region (Fig. 5). In IgG3, residues 330 and 331 are Ala and Pro, respectively; in IgG4, these are replaced by Ser-Ser, and in mouse IgG2b, by Ser-Pro. In as much as the affinity of IgG2b can be increased to the level of IgG3 by a single amino acid substitution in the hinge-link region, the presence of serine at position 330 does not appear detrimental to receptor interaction. To evaluate the role of Ser(331) in the binding affinity for  $Fc\gamma R_1$ , a second substitution was made in the mutant IgG4(Leu[234]) generating IgG4(Leu[234], Pro[331]); a reciprocal mutation was generated in IgG3 yielding IgG3(Ser[331]).

IgG3(Ser[331]) is reduced in affinity for  $Fc\gamma R_1$  by a factor of 10, indicating that Pro(331), like Leu(234) and Leu(235), is important for high affinity receptor association (Table 2). Curiously, the double-mutant IgG4(Leu, Pro) shows no improvement in  $K_a$  relative to IgG4(Leu[234]), and in fact appears reduced in affinity, indicating that the appropriate combination of factors for high affinity binding still has not been achieved.

Another feature of the IgG4 molecule that might contribute to its reduced affinity relative to IgG3 is its hinge, which is relatively rigid. To evaluate the possibility that the mutant IgG4(Leu[234]) is reduced in  $K_a$  relative to IgG3 due to the stiff nature of its hinge region, the hinge of IgG4(Leu[234]) was replaced with a  $\gamma 3$  hinge, yielding the antibody IgG4(Leu[234])H $\gamma 3$ . As indicated in Table 2, however, the



**Figure 5.** Graphic representation of the Fc region of IgG.  $C_H2$  is shown in yellow,  $C_H3$  and the  $C_H2$ -linked carbohydrate are in white. Pro 238 is red, Pro 331 is magenta, with the rest of the loop from 328 to 333 shown in blue. Ala 327 is light blue-green. The coordinates are from Diesenhofer (16); the graphic was made using the MacImdad computer graphics package. The two Fc regions face in opposite directions, therefore, the residues visible on the right domain are invisible on the other side of the left domain.

hinge substitution does not improve binding affinity. Like the double-mutant IgG4(Leu, Pro), IgG4(Leu[234]), H $\gamma$ 3 exhibits a  $K_d$  value reduced 10-fold from the level of IgG3.

There are several conclusions that can be drawn from these results. The association of IgG with Fc $\gamma$ R<sub>1</sub> is directly influenced by multiple amino acid residues in the C<sub>H</sub>2 domain. These residues are not limited to the peptide segment defined by Duncan et al. (15), but are also found in a hinge-proximal bend formed between two  $\beta$  strands in the globular domain structure (Fig. 5). Since these residues all lie within close proximity, they may all contribute directly to receptor association. In addition, the hinge region appears to modulate receptor affinity. Since molecules bearing hinges varying widely in length and sequence all show reasonable binding affinity ( $K_d > 10^8$  M<sup>-1</sup>), it seems likely that the hinge does not play a direct role in receptor interaction but instead exerts a conformational effect and, by controlling the spacing of the Fab and Fc regions, determines the accessibility of the receptor binding site on the Fc.

## Discussion

The mutational analysis used in this work to investigate the interactions of IgG with its high affinity receptor represents a powerful technique for discerning the contributions made by various regions of the molecule to overall effector function activity. The anti-DNS chimeric antibody system makes this approach possible by linking the cloned human  $\gamma$  and  $\kappa$  constant region genes to their respective murine H and L chain variable region genes in an expression system that yields mAb production in transfected myeloma cells. The relative facility with which these cloned genes can be manipulated and expressed provides the unique opportunity to focus attention on a particular region within the IgG molecule, while operating in the context of the whole polypeptide. The use of mutagenesis to isolate regions functionally without requiring physical isolation (e.g., by fragmentation) allows for the correlation of structure with function without artifacts introduced by loss of native conformation. Chimeric Igs have normal Ig function and, indeed, the values we obtain for the association constants of IgG1, -3, and -4 binding to the monocyte receptor on U937 cells are in agreement with previous published reports (23).

The relative Fc $\gamma$ R<sub>1</sub> affinities of the mutant antibody proteins examined here offer a number of insights into the role of IgG structure in effector function. The association constants of IgG2 and IgG3 are unaltered by exchange of the C<sub>H</sub>3 domain between the subclasses, indicating that the structural basis for the nonbinding phenotype of IgG2 does not reside in the COOH-terminal domain. Likewise, the features of the IgG3 molecule that confer on it high affinity for Fc $\gamma$ R<sub>1</sub> are not contained within the C<sub>H</sub>3 domain. Admittedly, this analysis is limited to localizing the structural differences between proteins responsible for variations in functional efficiency. To the extent that the C<sub>H</sub>3 domains of IgG2 and IgG3 contribute equally to receptor binding, the degree to which they contribute may not be determined. Comparison of the amino acid sequences of the C<sub>H</sub>3 domains in

IgG1, -2, -3, and -4 (14) reveals 11 positions at which the four subclasses are not identical. However, only one site of sequence difference between subclasses, located three residues from the COOH terminus of the C<sub>H</sub>3 domain, is likely to be solvent accessible. As this residue is shared by IgG1, -2, and -3 (Pro) but differs in IgG4 (Leu), it could conceivably contribute to the reduced affinity of IgG4. Though it is impossible to rule out this contribution without specifically substituting this residue, its position relative to C<sub>H</sub>2 residues, shown by us and others (15) to be critical to Fc $\gamma$ R<sub>1</sub> binding, makes it an unlikely candidate for a role in receptor binding. We therefore conclude that the C<sub>H</sub>3 domain of IgG does not contribute to the Fc $\gamma$ R<sub>1</sub> affinity differences displayed by the subclasses.

The hinge region imparts segmental flexibility to IgG. Among the human IgG subclasses, IgG3 stands out as possessing the most radically different hinge structure and displays the greatest segmental flexibility (27); the exceptional flexibility of IgG3 is mediated by that region encoded by the first (unique) hinge exon (22). The requirement of a hinge per se for full effector function activity has been demonstrated for IgG1 using the hinge-deleted myeloma variants Dob and Lec; in these proteins, the close contact of C<sub>L</sub> and C<sub>H</sub>2 domains emphasizes the role of the hinge as a spacer between Fab and Fc. Likewise, in IgG3, deletion of the hinge region altogether abrogates Fc $\gamma$ R<sub>1</sub> binding (as well as complement fixation [22]). Unlike IgG1, however, IgG3 appears to require a long spacer for full effector function. Hence, IgG3 bearing a hinge of 17 amino acids encoded by hinge exon 1, but lacking the rigid spacer encoded by the repetitive exons 2, 3, and 4, is reduced in affinity for Fc $\gamma$ R<sub>1</sub>. We propose that the added Fab flexibility mediated by the IgG3 upper hinge would permit close approximation of the Fab arms and the Fc $\gamma$ R<sub>1</sub> contact site were it not for the added distance provided by the extended hinge; thus, high Fab flexibility in IgG3h<sub>1</sub> may sterically compromise Fc receptor access to its binding site. It should be noted that the amino acid composition of hinge segment 1 is different from that of the reiterated segments normally abutting the hinge-like region, however, five amino acids most proximal to the hinge-link are identical in all four hinge segments, and in fact, represent the conformationally constrained spacer unit (Cys - Pro - Arg - Pro). Interestingly, when the hinge reaches a certain length, added spacing appears detrimental to effector function, perhaps because of flexion in the polyproline helix. Hence, IgG3 with a hinge of 117 amino acids is compromised in its affinity for Fc $\gamma$ R<sub>1</sub>, as well as in its ability to consume complement (22).

The role of the C<sub>H</sub>1 domain in the association of IgG with human high affinity receptor appears to be limited to maintenance of overall quaternary structure since the Fc region alone shows full binding capacity. The requirement for this domain for full strength binding to Fc $\gamma$ R<sub>1</sub> may represent the effects of steric hindrance on antibody receptor interaction by the C $\kappa$  domain, without an analogous C<sub>H</sub> domain with which to pair in these mutants. Both IgG $\Delta$ C<sub>H</sub>1 and IgG3 $\Delta$ C<sub>H</sub>1 show reduced affinity for Fc $\gamma$ R<sub>1</sub> with IgG1 $\Delta$ C<sub>H</sub>1, showing the most profound reduction. The pattern of H-L disulfide bridging differs between IgG1 and IgG3:



the light chain in IgG1 bonds to the NH<sub>2</sub>-proximal cysteine residue in the hinge region, whereas in IgG3 this bond is to a cysteine residue in the C<sub>H</sub>1 domain. Based on these patterns, it seemed likely that the assembly of IgG3 would be more disturbed by the C<sub>H</sub>1 deletion than that of IgG1. In fact, the contrary was observed. Apparently, in IgG3ΔC<sub>H</sub>1, the L chain is able to use one of the 11 cysteine residues in the extended hinge to form the H-L bridge. Electromicrographic studies (V. Schumaker, unpublished results) have shown that the extended hinge of IgG3 is about the same size as a domain, and apparently the hinge can assume some of the roles played by C<sub>H</sub>1 in the C<sub>H</sub>1-deleted molecule. In IgG1ΔC<sub>H</sub>1, on the other hand, the hinge is much shorter, and apparently Cκ cannot assume a conformation suitable for disulfide bonding with this hinge. Hence, IgG3ΔC<sub>H</sub>1 is secreted predominantly as covalent H<sub>2</sub>L<sub>2</sub> tetramers, whereas IgG1ΔC<sub>H</sub>1 is secreted primarily as covalently linked H chain dimers.

The IgG region most clearly implicated in direct binding of the human FcγR<sub>I</sub> is the C<sub>H</sub>2 domain. Binding phenotype is transferred with this domain in exchanges between binding and nonbinding subclasses. Hence, IgG2 bearing the C<sub>H</sub>2 domain of IgG3 is improved at least 300-fold in receptor affinity. Though the mutant 2232 did not display IgG3-level FcγR<sub>I</sub> affinity, this is not entirely surprising considering the conformational contributions to Fc function made by the hinge region.

Intradomain substitutions within C<sub>H</sub>2 itself identify two regions of the primary structure as contributing to the differential subclass affinities for FcγR<sub>I</sub>. The pair of leucine residues in the hinge-link region (positions 234 and 235, EU numbering system) shared among all high affinity IgGs examined are critical to Fc receptor association, since substitution of either of these residues with the amino acid present in subclasses of reduced affinity results in reduction of K<sub>i</sub>. Leu (bearing a branched hydrocarbon side chain) is replaced by a Glu residue (bearing a negatively charged side chain) at position 235 in muIgG2b. The introduction of a charged residue at this site may interrupt hydrophobic interactions critical to receptor association, resulting in the severely compromised binding affinity of this Ig (>100-fold reduced relative to the strongly binding subclasses, huIgG1, -3, muIgG2a). In human IgG4, on the other hand, Leu is replaced by Phe (bearing an aromatic side chain) at position 234. The introduction of a bulky aromatic group in this position may cause steric hindrance of the IgG-FcγR<sub>I</sub> interaction, however, the nonpolar environment of the region is maintained. This disturbance appears less severe than that caused by the Glu for Leu substitution, since IgG4 is reduced in binding affinity only 10-fold relative to the strongly binding subclasses.

Residue 331 (Pro in IgG1 and -3), near the COOH-terminal end of the domain sequence, also appears directly involved with receptor interaction, since FcγR<sub>I</sub> association is highly sensitive to substitution at this site. Though separated by 96 residues in linear sequence, these two sites are in fact quite

close in space due to the tertiary structure of the C<sub>H</sub>2 domain (Fig. 5). Examination of the crystallographic structure of FcγR<sub>I</sub> by computer graphics using the atomic coordinates of Deisenhofer (16) reveals that residue 331 lies in a peptide loop between two β strands, at the hinge-proximal end of the domain. Though the hinge-link region is not resolved due to a high degree of disorder at this site in the crystal structure, Pro(331) folds into close proximity with Pro(238) (the first resolvable residue in the Fc) of the hexapeptide L - L - G - G - P - S discussed earlier (<11 Å between α-carbon atoms) (see Fig. 5). Our findings represent the first experimental evidence that the hinge-link region and hinge-proximal bend may function together in binding the human high affinity Fc receptor. These two regions may serve as contact points for receptor association, or alternatively, one region may be critical for stabilizing peptide conformation in the other region, allowing FcγR<sub>I</sub> proper access. A puzzling issue is why the Phe(234) and Pro(331) substitutions do not restore maximal binding to IgG4. IgG2 and IgG4 differ from IgG1 and IgG3 at amino acid 327 (light blue-green in Fig. 5); this residue is Gly in IgG2 and -4, and Ala in IgG1 and -3. Residue 327 is not completely conserved for it is Asp in murine IgG2b, and that substitution does not appear to compromise FcγR<sub>I</sub> binding. The Ala, Gly substitution would be highly conservative, however, the Gly substitution may impart additional mobility to the 328-333 bend and this mobility may impair binding.

Our results do not delimit the extent of FcγR<sub>I</sub>-interacting residues. Indeed, Woof et al. (25) have pointed out that the COOH-terminal 20-30 residues of C<sub>H</sub>2 show numerous sites of identity between IgGs with high affinity for FcγR<sub>I</sub>. Most of these sites extend along two of the three β strands in the Y-face of the domain, with Pro 331 falling at the bend between the strands. Thus, with regard to proximity of these residues to the hinge-link region, residue 331 and those neighboring it (Fig. 5) appear the most likely candidates for receptor interaction. Clearly, more site-specific substitution will be required to further delimit the residues critical to high affinity binding.

Clearly, multiple features of the IgG molecule influence its interaction with FcγR<sub>I</sub>. In the divergent evolution of the four human IgG subclasses, factors making negative and positive contributions to binding have been sorted to yield the present binding phenotypes. Reduction of affinity may follow a single alteration in a structural feature required for receptor association, as demonstrated by the Glu(235) or Ser(331) substitutions of IgG3. On the other hand, increasing binding affinity may require an appropriate combination of features (e.g., the high segmental flexibility of IgG3 matched with the long hinge of this molecule) before an observable improvement is achieved. This may explain the seemingly paradoxical results obtained with the IgG4 substitutions mutants. Engineering antibodies with desired receptors binding properties may require a combination of the proper structural alterations.

This work was supported by U.S. Public Health Service grant CA-16858 from the National Institutes of Health and by a grant from the American Cancer Society (IM-550). S. M. Canfield is supported by NIH grant GM-07367 under the Medical Science Training Program.

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Received for publication 30 November 1990 and in revised form 26 February 1991.

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